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Rapid Intracellular Turnover of Adenovirus 5 Early Region 1A Proteins

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The half-life of the adenovirus 5 early region 1A (E1A) proteins was examined in productively infected and transformed cells. In HeLa cells infected with adenovirus 5, the half-life of the E1A proteins was approximately 30 min; in the transformed 293 cells, the constitutively expressed E1A proteins had a half-life of approximately 120 min. In HeLa cells, the E1A proteins produced by an adenovirus mutant that expresses only the 13S mRNA had a half-life of about 35 min; E1A proteins produced by a mutant that expresses only the 12S mRNA had a half-life of about 80 min. This difference in the stability of these two classes of E1A proteins helps explain why the steady-state level of the 12S class is usually equal to or greater than that of the 13S class, despite the fact that the concentration of the 13S mRNA is about four times greater than the concentration of the 12S mRNA.

Two highly related proteins of 289 and 243 amino acids are encoded by early region 1A (E1A) of the human adenovirus 5 (Ad5) (26). Each of these primary translation products is modified in a number of different ways by post-translational processes that do not alter amino- or carboxy-terminal sequences (4, 30, 34). These various modified forms of the E1A proteins can be separated on one- and two-dimensional protein gels. E1A proteins are necessary for the cellular transformation of rodent cells in vitro (9, 11, 15, 17, 33). During infection by adenovirus, the E1A 289-amino-acid protein induces the expression of all other early viral genes (2, 16, 23, 24, 28). This protein also stimulates transcription from nonviral genes introduced into cells by infection or transfection (5, 12), and it can stimulate transcription of an integrated early adenovirus gene (3a). The E1A 243-amino-acid protein is required for efficient viral replication in growth-arrested human cells (22). We are investigating the mechanisms by which the E1A proteins can exert these effects on cell metabolism and transformation. Although the E1A mRNA levels are high in infected cells, the E1A proteins are present in low concentrations (14). The low protein levels could be explained by translational regulation or rapid turnover of the proteins. We report here an investigation of the turnover of the E1A proteins with immune precipitation with specific antiserum in pulse-chase labelings of productively infected HeLa cells and in the transformed 293 human cell line.

To achieve maximal sensitivity, most of our pulse-chase experiments in productively infected HeLa cells were performed during the period of infection when E1A protein synthesis was at its peak. However, similar E1A protein half-lives were observed at earlier times in infection (see below). To determine the period of peak E1A protein synthesis we analyzed E1A protein levels in HeLa cells infected with Ad5 between 4 and 48 h postinfection (p.i.) with Western immunoblots (Fig. 1). Maximal E1A protein concentrations (and therefore protein synthesis since the E1A proteins are very unstable; see below), occurred between 14 and 18 h p.i. Incorporation of [^{35}S]methionine (1,160 Ci/mmol; Amersham Corp.) at a concentration of 10 μCi/ml was linear for at least 2 h at 14 h p.i. (data not shown). These infection and labeling conditions were used for the pulse-chase experiments described below.

Quantitative extraction and immune precipitation of the E1A proteins was essential for the success of these analyses. Five 100-mm plates infected with Ad5 were analyzed for each time point, so that extraction could be performed in volumes that reproducibly gave nearly quantitative solubilization of the E1A proteins. The cells were lysed by the procedure of Manley et al. (21), except that only 0.7 packed-cell volume of saturated $(NH_4)_2SO_4$ was added, and 100 U of Trasylol (Mobay Biochemical) per ml was added. The lysates were ultracentrifuged for 1 h (100,000 × g) as described previously (32), the supernatant was dialyzed against phosphate-buffered saline, and a sample was mixed with an equal volume of Laemmli gel sample buffer. The pellet fraction was rinsed with 0.1% Nonidet P-40–2 mM $CaCl_2$–20 mM Tris (pH 8.8), suspended in the same buffer, digested with micrococcal nuclease, DNase I, and RNase A as described previously (6), and mixed with an equal volume of Laemmli gel sample buffer for analysis by Western blotting. Approximately 95% of the E1A proteins were solubilized as estimated by densitometric scanning of the immunoblot (Fig. 2). Note that the material from 10 times as many cells from the pellet fraction was analyzed to visualize the E1A proteins. Quantitative transfer of the proteins occurred, as determined by Coomassie blue staining of the acrylamide gel after transfer (data not shown). Similar extraction recoveries of the E1A proteins were obtained from 293 cells (data not shown).

The Ad5-infected cells were pulse-labeled at 15 h p.i. in Dulbecco modified Eagle medium minus methionine plus 2% dialyzed newborn calf serum and 10 μCi of [^{35}S]methionine (1,160 Ci/mmol) per ml in a volume of 5 ml. After 90 min of labeling, the samples were harvested (pulse) or chased by adding 5 ml of medium to each plate (a >20,000-fold excess of cold methionine). Five plates were processed as described in the legend to Fig. 2 for each time point; the chase times were 30, 60, or 120 min (Fig. 3). The supernatants were dialyzed against phosphate-buffered saline at 4°C. Extracts from 5 × 10^6 cells were precleared by incubating with 25 μl of preimmune serum for 4 h and with 250 μl of 10% fixed *Staphylococcus aureus* for 30 min, and then the immune complexes were removed by ultracentrifugation as described previously (32). The clarified supernatants were

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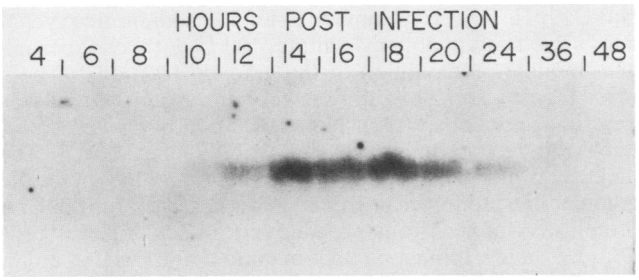

FIG. 1. Time course of E1A protein levels. HeLa cells were infected with Ad5 at a multiplicity of infection of 10, harvested at the time points indicated, boiled in Laemmli gel sample buffer (18), and analyzed by Western immunoblotting with an antiserum to an E1A-trpE fusion protein and ^{125}I-labeled *S. aureus* protein A (32). Extract from 5×10^6 cells was loaded in each lane.

incubated with anti-E1A-fusion protein serum in antibody excess, and immune precipitates were prepared and analyzed on a 10% polyacrylamide gel as described previously (32). Control experiments demonstrated that antibody was in excess and immune precipitation was quantitative. Figure 3 shows that the [^{35}S]methionine label in E1A proteins chased rapidly, consistent with an earlier report (29). No precursor-product relationship among the various electrophoretic forms of the E1A proteins was detected in these experiments.

The autoradiogram in Fig. 3 and another from a duplicate experiment were scanned by densitometry (Fig. 4). The half-lives of the E1A proteins from these two experiments were estimated to be 36 and 24 min, respectively. Similar results were obtained with E1A proteins labeled 6 h p.i. (data not shown).

Several factors can influence the determination of the half-life of the E1A proteins. In a pulse-chase labeling of cultured cells the specific activity of the total intracellular pool of the labeled precursor remains higher than that in the media,

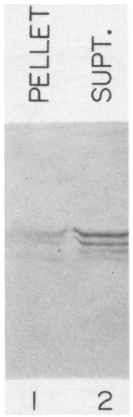

FIG. 2. Extraction of E1A proteins. Five 100-mm plates of HeLa cells were infected with Ad5 at a multiplicity of infection of 10 and harvested at 17 h p.i. The E1A proteins were solubilized as described in the text. One-fifth of the pellet fraction and 1/50 of the supernatant were electrophoresed on 10% polyacrylamide gels and analyzed by Western immunoblotting as in Fig. 1, except the nitrocellulose was blocked with 9.6% nonfat dry milk and the first antibody was detected with goat anti-rabbit immunoglobulin G–horseradish peroxidase conjugate and the Immun-Blot assay kit (Bio-Rad). Lanes: 1, pellet from 10^7 cells; 2, supernatant from 10^6 cells.

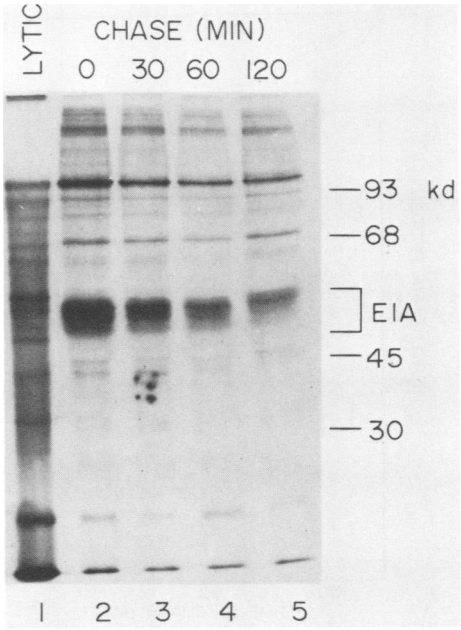

FIG. 3. Pulse-chase labeling of E1A proteins in HeLa cells. HeLa cells were infected in parallel with those described in Fig. 2 and pulse-labeled at 15 h p.i. as described in the text. The pulse sample was harvested at 90 min p.i. without the addition of Dulbecco modified Eagle medium (0-min chase). The chase samples were harvested at 30, 60, and 120 min after adding the medium, as indicated. The E1A bands are indicated. Lane 1 contains ^{35}S-labeled proteins from late in a lytic infection. The numbers along the side indicate positions of ^{14}C-labeled protein standards (Amersham).

despite the presence of unlabeled precursor added during the chase (7, 27). These labeled precursors can then be reutilized during the chase period. This could lead to an overestimate of the half-life of the E1A proteins. In addition, the antiserum used in this study recognizes both the 289- and 243-amino-acid proteins encoded by E1A (32). Thus the half-life determination reported here is for both E1A proteins.

To examine the turnover of the individual E1A proteins, we used the mutants pm975 and *dl*1500, which produce only the 289- and 243-amino-acid proteins, respectively (22, 23). Pulse-chase experiments were performed and quantitated exactly as for wild-type Ad5 infection (Fig. 4). The half-life of each of the individual E1A proteins was also very short, approximately 34 min for the 289-amino-acid protein and 79 min for the 243-amino-acid protein. The 13S mRNA, which encodes the 289-amino-acid protein, is present at approximately four times higher concentrations than the 12S mRNA (3). However, the 243-amino-acid protein level is equal to or greater than the 289-amino-acid protein level (6, 13, 30, 34). The difference in the stability of the two E1A proteins, as measured here, may contribute to the increased steady-state level of the 243-amino-acid protein relative to the 289-amino-acid protein. Also, in each of these mutants, the absence of one of the E1A proteins may alter the half-life of the remaining E1A protein, relative to its half-life in the wild-type infection.

The cellular tumor antigen p53 has a very short half-life, approximately 30 min, in nontransformed 3T3 cells (25). However, in simian virus 40-transformed cells the half-life of p53 is greater than 22 h (25). It has been postulated that this is due to a stabilizing effect exerted by a physical association between p53 and the simian virus 40 large tumor antigen (25).

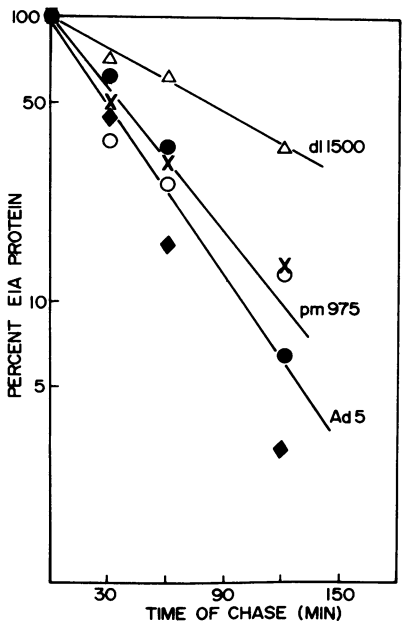

FIG. 4. Half-life of E1A proteins in wild type- and mutant-infected HeLa cells. The autoradiograms of Ad5-infected cells in Fig. 3 (×) and a duplicate experiment (◆) were scanned with a Hoefer scanning densitometer. The E1A peaks were cut out, and the peak masses were normalized to a value of 100% for the pulse sample in each experiment. The data from both experiments were used in a linear regression analysis to obtain the half-life curve designated Ad5. From this curve a half-life of 28 min was determined. Similarly, the data from two pulse-chase experiments with pm975 (○ and ●) were used to obtain the indicated curve for pm975. A pulse-chase labeling of *dl*1500 and similar analysis yielded the half-life curve (△) for the *dl*1500 E1A protein.

To see whether the turnover of the E1A proteins is altered in transformed cells, we examined the half-life of the E1A proteins constitutively expressed in the human 293 cell line. These cells have integrated DNA from early region I of Ad5 and express E1A and E1B mRNAs and proteins (1, 2, 10, 20). Pulse-chase labelings, extraction, immune precipitation, and quantitation of the E1A proteins were performed as for the lytic infections described above. Initially we used chase periods of 0, 30, 60, and 120 min, as in productive infection of HeLa cells. Little decrease in the amount of E1A proteins was observed (data not shown), so we used chase periods of 4 and 8 h to better quantitate the half-life in 293 cells (Fig. 5). Control immune precipitations with preimmune rabbit serum indicated that there was some background radioactivity precipitated in the E1A region in these 293 cell labelings, and so this radioactivity was subtracted to obtain the half-life curve shown in Fig. 6. Two additional duplicate experiments were performed, with chases of 4 and 7 h, and are also shown in Fig. 6. The half-life of the E1A proteins in the 293 cells was approximately 120 min. This longer half-life of the E1A proteins in the 293 cells could simply reflect a difference in overall rates of protein turnover in 293 cells versus HeLa cells. Alternatively, other viral genes expressed during a productive infection of HeLa cells could lead to more rapid turnover. Another possibility is that the E1A proteins are associated with other protein(s) in 293 cells, which increases their half-lives.

To examine the latter possibility, we performed glycerol gradient sedimentation in RIPA buffer (0.15 M NaCl, 0.01 M $NaPO_4$ [pH 7.0], 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 100 U of trasylol per ml) and immune precipitation of the gradient fractions of both pulse-labeled and chased E1A proteins from 293 cells as described previously (32). No association of the E1A proteins with other proteins was apparent (data not shown). The majority of the E1A proteins sedimented near the top of the gradient in a monomer position, as we observed for the E1A proteins extracted from productively infected HeLa cells (32). However, it must be kept in mind that the RIPA buffer may disrupt some weak, but nonetheless biologically significant, protein-protein interactions.

Most eucaryotic proteins examined have half-lives on the order of 2 or 3 days, although a number of enzymes have shorter half-lives (for review, see reference 8). Those enzymes with short half-lives represent important regulatory points in cellular metabolism (8). In an analogous manner, the short half-lives of the E1A proteins during productive infections may be significant with respect to the requirement of the E1A 243-amino-acid protein for viral replication in growth-arrested cells (22) or the transcription stimulating activity of the 289-amino-acid protein (2, 16, 23, 24, 28). Perhaps the E1A proteins are rapidly degraded as a consequence of their mechanism of action.

The gene product of the c-*myc* oncogene also has a short half-life of approximately 30 min (R. Eisenman, personal communication). The E1A proteins have been shown to function in transformation of fibroblasts similarly to the v-*myc* gene product, perhaps by providing an immortalization function (15, 19, 31). The fact that both the E1A proteins and the c-*myc* proteins turn over rapidly is intriguing. It is interesting that the half-life of the E1A proteins in the transformed 293 cells was about four times longer than in

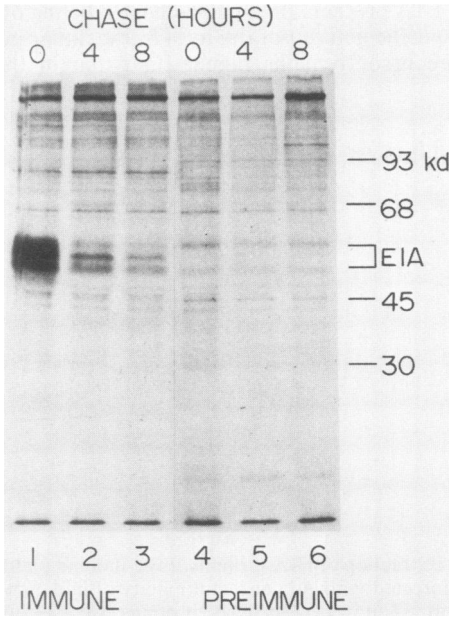

FIG. 5. Pulse-chase labeling of E1A proteins in 293 cells. Confluent monolayers of 293 cells were pulse-labeled for 90 min and chased for 0 (pulse), 4, and 8 h p.i., as described in the legend to Fig. 3. The cell proteins were extracted and immune precipitated with anti-E1A-fusion protein serum (lanes 1 through 3) or preimmune rabbit serum (lanes 4 through 6) and analyzed as described in the legend to Fig. 3. The E1A bands are indicated; the positions of ^{14}C-labeled protein standards are indicated by the numbers on the side.

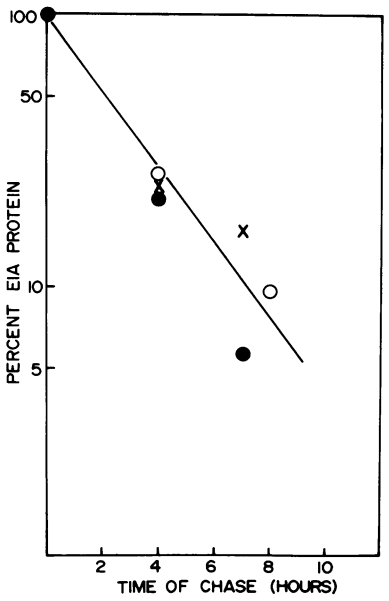

FIG. 6. Half-life of E1A proteins in 293 cells. E1A protein levels in the 293 cells were determined by scanning the autoradiogram in Fig. 5 (●) and autoradiograms from two additional duplicate experiments (○, ×) in which the chase periods were 4 and 7 h, respectively. The half-life of the E1A proteins in the 293 cells was calculated by linear regression analysis to be 120 min.

productively infected cells, although their turnover in 293 cells was still rapid for eucaryotic proteins. The levels of the E1A proteins in other transformed cell lines we have examined are much lower, precluding a measurement of their turnover. Thus, we cannot say whether this longer half-life of E1A proteins in 293 cells is a general property of E1A proteins in transformed cells. Elucidation of the functions of the E1A proteins may lead to an explanation for their remarkably short half-lives.

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